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THE CHRONOMUTAGENIC EFFECT OF DEUTERIUM OXIDE ON THE PERIOD AND ENTRAINMENT OF A BIOLOGICAL RHYTHM

H. BURGESS DOWSE AND JOHN D. PALMER

Department of Biology, New York University, New York, New York 10453

It is thought that underlying all circadian rhythms is a "biological clock" (Brown, Hastings and Palmer, 1970), and because of the near ubiquitous distribution of clock controlled rhythms throughout the living kingdom it is important to understand completely the machinery of this living horologue. Attempts to do this have been varied, and for the most part only incompletely—if at all—successful. One approach has been to subject rhythmic organisms to specific inhibitors of macromolecular synthesis, narcotizing agents, growth stimulants, sublethal doses of general metabolic poisons, and other kinds of sustained or pulsed chemical insults, in hopes of altering the clockworks and thus gaining some insight into its mode of operation. The great majority of substances used have produced no direct alterations on the rate at which the clock runs (Bühnemann, 1955; Hastings, 1960). The only generalization that can be drawn so far is that the living clock is virtually intractable to exogenous chemical manipulation.

Some of the most interesting and paradoxical results thus far obtained are with inhibitors of protein synthesis. For example, when the dinoflagellate, *Gonyaulax* was subjected to actinomycin-D, the bioluminescent glow rhythm was inhibited. Puromycin, another inhibitor of protein synthesis, also appeared to inhibit the rhythm but in essence inhibited all bioluminescence. Chloramphenicol had no effect on the length of period, but increased the amplitude of the rhythm many fold (Karakasian and Hastings, 1962; 1963). The other rhythmic processes known in *Gonyaulax* were not affected by actinomycin D. In studying the photosynthetic rhythm in enucleated *Acetabularia*, Sweeney, Tuffi and Rubin (1967) found that other than a reduction in amplitude, actinomycin-D, puromycin, and chloramphenicol had no observable effect on this organism's clock. Some of the strongest evidence thus far obtained with inhibitors of protein synthesis lies in the effect of cyclohexamide on the *Euglena* phototactic rhythm: it was found that the period of this rhythm increased as a function of the concentration of the inhibitor (Feldman, 1967). Because the period of the rhythm was altered, it is possible that cyclohexamide may be acting directly at the level of the clock and slowing it down.

To date, the only consistent effect obtained by the application of a single substance comes from studies using heavy water. Addition of D_2O to the culture medium of *Euglena* produced both phase and period alterations in the phototactic rhythm (Bruce and Pittendrigh, 1960). Period augmentation of the bean (*Phaseolus*) sleep-movement rhythm by D_2O was found by Bünning and Baltes (1963). The activity rhythms of the deer mouse, *Peromyscus* (Suter and Rawson, 1968), the African waxbill *Estrilda* (Palmer and Dowse, 1969), the rat (Richter, 1970), and an intertidal isopod, *Excirolana* (Enright, 1971) are simi-

larly affected. The following account describes several deuterium induced chronomutagenic (*Chrono* = time; *mutatio* = change; *genic* = producing) alterations in the spontaneous locomotor activity of the common laboratory mouse.

General Materials and Methods

The common laboratory mouse, *Mus musculus*, strain CF-1 (obtained from Carworth Farms, New City, New York) was used. This strain is known to display persistent rhythms (Dowse and Palmer, 1969). Each male was maintained in a cage (10 cm on a side) with free access to a running wheel (18 cm in diameter). Every revolution of the wheel activated a magnetic pen which recorded the event on an Esterline Angus chart recorder. Each self-cleaning cage held a 7–10 day supply of food (Purina rat chow) and H_2O or D_2O. A 99% solution of deuterium oxide, obtained from K & K Chemical Supply, was mixed with tap water to make solutions varying from 5 to 30% D_2O (in 5% increments).

The caged mice were maintained in light-proof, ventilated, sound attenuating walk-in chambers. The 20° C temperature varied adiurnally by less than 1° C. Cool-white fluorescent lamps were used as the illumination source, the light intensity being controlled by neutral density filters. Intensities were determined with a Weston model 756 photometer.

Data analysis and graphic presentation were patterned after the "array analysis" technique described in full elsewhere (Palmer, 1967). In brief, the amount of mouse activity for every hour of the day was calculated and a daily mean derived. A graph was then constructed in which each day was represented as an unshaded horizontal bar, subdivided longitudinally into 24 squares (one for each hour of the day), and all hourly activity values that equaled or surpassed the daily mean represented by blacking in the squares in the bar that corresponded to those hours. Thus represented, consecutive days are plotted one beneath the other (as in Figure 1). The net result was that minor fluctuations (*i.e.*, those below the daily mean) in the daily activity pattern were "filtered out" and do not appear on the graph, while the times of maximal activity are boldly emphasized along the otherwise unshaded bar. This method of graphic presentation condenses a great deal of data into a usable size (there are more than 96,000 mouse-hours of data used in this study) and emphasizes major trends—in particular, period estimates can be easily determined.

To quantify the effect of heavy water on the spontaneous locomotor rhythm, changes in the length of the period (measured by comparing the onsets of consecutive daily activity) were observed. The slope of lines superimposed over successive times of onset were fitted "by eye," after it was found that the "method of least squares" did not measurably improve the accuracy of this estimate (Dowse, 1971).

Experimental Rationale and Results

1. The effect of D_2O on the period of the persistent locomotor rhythm

After being subjected to a light-dark regimen of 12 hours of light (8 foot candles) alternating with 12 hours of darkness [abbreviated as LD 12:12 (8) according to the convention of Aschoff, Klotter and Wever (1965)] for 1–2 weeks, mice were switched to a constant light intensity (LL) of 0.2 foot candles for an interval

TABLE 1

Period lengthening in percent of the mouse persistent locomotor activity rhythm by D_2O consumption.

D_2O	Mice in LL (0.2 ft. c.)				
	Mouse No.	Period before D_2O	Period during D_2O	Per cent difference	$\bar{X}$ difference at each dose
5%	1	24.38	24.50	.49	1.04
	2	23.00	23.30	1.30	
	3	24.25	24.57	1.32	
10%	4	24.38	24.83	1.84	2.03
	5	24.37	24.91	2.21	
15%	6	23.00	24.00	4.35	3.88
	7	23.75	24.91	4.88	
	8	24.69	25.29	2.43	
20%	9	23.38	24.86	6.33	5.67
	10	24.23	25.14	3.75	
	11	23.77	25.14	5.76	
	12	23.00	24.57	6.83	
25%	13	24.20	25.50	5.37	5.26
	14	24.25	25.50	5.15	
30%	15	23.77	25.70	8.11	7.31
	16	23.20	25.09	8.13	
	17	24.47	25.86	5.68	
	Mice in DD				
20%	18	23.17	25.14	5.76	5.67
	19	23.62	24.86	5.24	
	20	24.00	25.14	4.75	
	21	23.38	25.00	6.92	
	Blinded mice in DD				
10%	22	24.44	25.00	2.29	2.55
	23	24.43	25.00	2.33	
	24	23.75	24.47	3.03	
20%	25	24.00	25.00	4.16	5.01
	26	24.30	25.44	4.69	
	27	24.00	25.44	6.00	
	28	24.00	25.25	5.20	

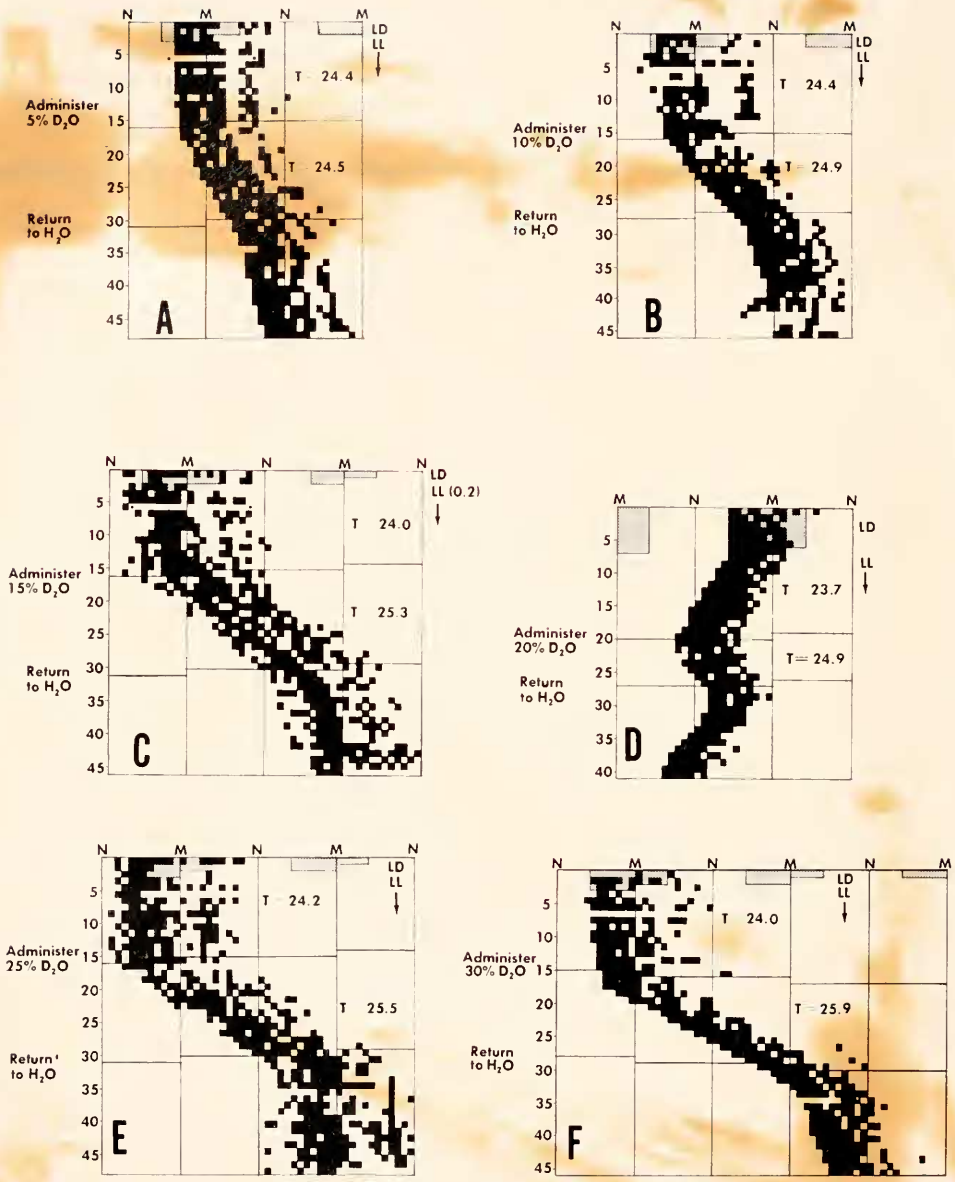
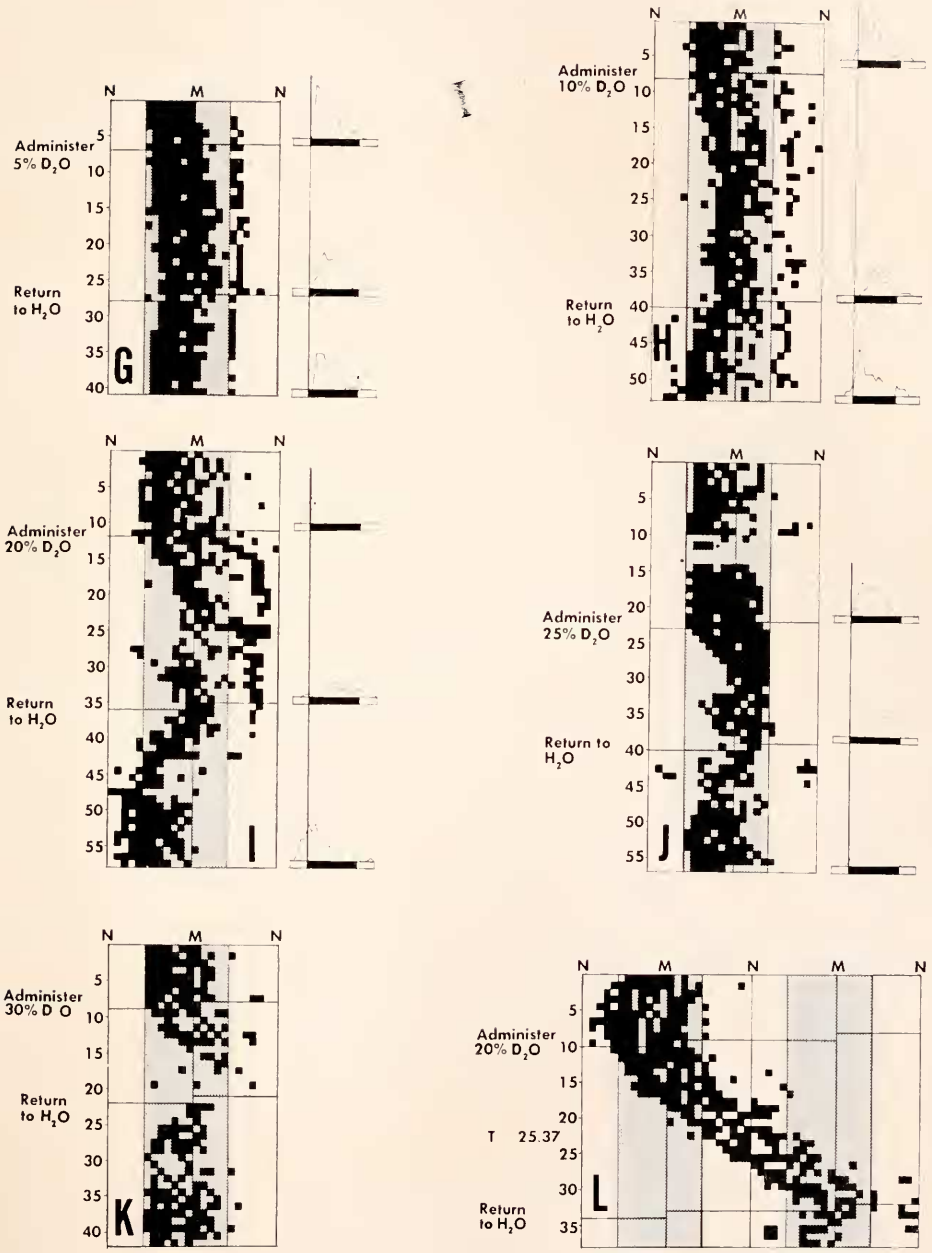


FIGURE 1. The role of D_2O consumption on the period (A-F) and phase (G-L) of the mouse circadian locomotor rhythm. IA-F are representative examples of mice which were initially kept in LD 12:12 (8), the last few days of which are indicated by LD notations (stippling signifies times of darkness) and then switched to LL (0.2) for the remainder of the study. The intervals of D_2O ingestion, with concentrations, are indicated on right hand ordinate. T = period estimate in hours. Heavy black bands indicate times of maximum spontaneous running (described in detail in text). Note that if one tries to display circadian frequencies on a 24 hour abscissa, a time comes when the peaks will "disappear" off one side



of the graph. To prevent this, and improve graphic clarity, the abscissas are extended beyond 24 hours as necessary. 1G-K are representative examples of the degree of phase delay or period augmentation (entrainment breakaway) displayed by mice given various concentrations of D_2O (as indicated) in LD 12:12 (8). Average form-estimate curves given on right ordinate. 1L is a representative example of a mouse fed 20% D_2O in LD 12:12 (0.2).

of time (1–2 weeks) sufficient to obtain an accurate estimate of the period of their persistent locomotor rhythm. At the end of this treatment, their water bottles were substituted with ones containing D_2O in concentrations varying between 5 and 30% (in 5% increments). The mice then consumed these mixtures for the next 1–2 weeks (until a reliable period estimate could be made) and were returned to proteated water.

In all cases, D_2O increased the period of the activity rhythm, the response being a function of the deuterium concentration consumed (Table I; Figs. 1A-F and 2), with a maximum increase of about 7.4% at 30% D_2O. On return to H_2O, the period reverted to a value identical with, or close to, the pre-deuterium value, although several days were usually required—the delay presumably due to a prolonged "wash out" time. The amount of activity was also altered by D_2O ingestion [Dowse, 1971; and since confirmed by Hayes and Palmer (unpublished experiments)]. [Palmer and Goodenough (unpublished) have obtained a similar response with the waxbill, *Estrilda*].

It is known that the period of most persistent biological rhythms is a function of the intensity of the ambient constant light, *e.g.*, the mouse activity rhythm has been shown to have a period of 23.5 hours in a constant light intensity of less than 0.1 foot candle, and increases with increasing light to 25.5 hours at 20 foot candles (Aschoff, 1960). The possibility exists that deuteration may be lengthening the period of this rhythm indirectly, *i.e.*, by modifying the mouse photoreceptors or optic center so that under the influence of different concentrations of heavy water the mice "see" a constant light level as different intensities. If this is the case, one would expect the period to change—probably in a regular way—with increasing concentrations of D_2O, *i.e.*, just as we found. This possibility was examined in 2 ways: by maintaining 4 mice in constant darkness (DD) during exposure to D_2O, and to eliminate any possibility of stray or residual light in the experimental chamber, by surgically blinding 7 other mice. Bilateral enucleation was performed surgically. Diabutal was used as the anesthesia and bleeding was minimized with Gelfoam. The mice were allowed one week postoperative recovery before observing their activity patterns in the running wheels.

All 4 mice in DD were subjected to 20% D_2O for 17 days. The results (Table I) are added to Figure 2. As can be seen, these data do not differ from those obtained similarly in constant dim light.

Three blinded mice were given 10% D_2O for 13 days, while the other 4 received 20% for 16 days. These data have also been added to Table I and Figure 2. Again there is a clear lengthening of the circadian period which is completely compatible to the response of intact mice in LL.

2. Role of deuterium on the entrainment of rhythm by LD cycles

LD cycles are the single most effective zeitgeber known; so influential are they that the biological clock can be entrained even to artificially shortened "days," *e.g.*, 11 hours of light alternating with 11 hours of darkness. The following experiments were performed to observe the entrainability of a deuterium influenced rhythm by LD cycles.

Thirty-three mice were exposed to LD 12:12 (8) for two weeks, after which deuterium was added to their drinking water to produce concentrations varying be-

tween 5 and 30% D_2O. After observation intervals of up to 33 days, the mice were returned to proteated water again. Three classes of responses were observed: (i) no effect, (ii) a change of the phase relationship with the ambient LD cycle (always a phase delay) Figure 1H), or (iii) a "breakaway" response in which the rhythm was no longer entrained by the LD cycle (Fig. 1K). The concentration of D_2O consumed, to a large extent determined the degree of the phase delay, or the destruction of the entrainability of the LD cycle.

Figure 1G shows the data from a mouse subjected to 5% D_2O. The mouse adjusted the onset of its activity to occur 1 to 2 hours after the onset of darkness.

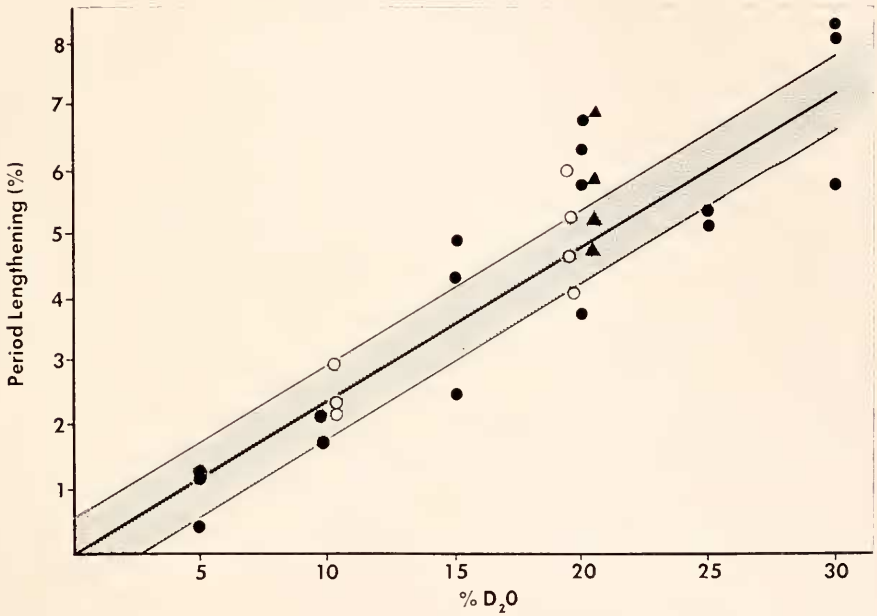


FIGURE 2. Summary of the chronomutagenic effect of D_2O on the period of the mouse circadian activity rhythm. Each point represents the percent difference between the period lengths of individual mice before and during deuterium consumption. The solid circles represent normal mice in LL (0.2), the open circles indicate blinded mice, and the triangles intact mice in DD. The curve was fitted for the mice in LL by the method of least squares (slope = 0.248; Pearson coefficient of linear correlation = 0.914; $S_y = 1.02\%$). The stippling signifies ± one standard error of the estimate.

Of the 7 mice tested at 5%, 4 delayed their phase at least 1 hour, and 3 were not affected. None broke away from the LD cycle.

The 10% dose caused a phase delay of between 2 and 3 hours in 7 of the 8 mice tested, the 8th being unaffected after a short exposure. Figure 1H shows a plot of a representative phase-delayed mouse given the 10% dose.

At 15%, 4 of the mice delayed their phase between 3 and 4 hours; one other adopted a circadian period of 25.7 hours, thus appearing to "ignore" the ambient LD cycle.

Of the 5 mice tested at 20%, one was unaffected by the treatment, 2 showed

clear phase delays of about 5 hours, one broke away from the LD cycle, and one showed an unclear response. Figure 1I shows one of the phase delayed mice.

At 25% D_2O 2 mice broke away from the zeitgeber, one remained entrained with a 6-hour phase delay (Fig. 1J), and one became arrhythmic.

At 30%, 2 of the mice tested were not entrained by the LD cycle (Fig. 1K), and a third test animal became inactive at this high dosage.

In summary, the data clearly show increasing perturbations of the relationship between the LD cycle and the rhythms as the dose of deuterium becomes greater. These effects are manifested at the lower concentrations by larger and larger phase lags, and by an increase in the ratio of breakaway mice to phase delayed mice at the higher concentrations.

3. The role of light intensity in LD entrainment of deuterated mice

While the intensity of light used to entrain rhythms need not be particularly bright (Hastings, 1964) it has been demonstrated that increasing the intensity of light used in artificial LD cycles improves its effectiveness as an entraining agent (Wilkins, 1960). One of the clearest trends emerging from the above LD experiments is the apparent decrease in the entraining influence of LD cycles with increasing deuteration of mice. Presumably, the intensity of the LD zeitgeber should also play a role in the breakaway-or-rephase response, so the following two experiments were performed. Six mice were maintained in LD 12:12 (0.2) while a second set of 8 were exposed to LD 12:12 (80). Two of the former and 3 of the latter mice served as controls; the others were given 20% D_2O.

All four mice subjected to LD 12:12 (0.2) and 20% D_2O failed to be entrained by the ambient LD cycle (Fig. 1L). Of the 5 mice given 20% D_2O in LD 12:12 (80), all were entrained to the zeitgeber and all displayed phase delays (similar to that in Figure 1I) of about 5 hours. All control animals remained strictly entrained to their LD cycles.

These data demonstrate that increasing the intensity of light in an LD cycle produces an overriding entraining influence on the phase or period altering effect of D_2O.

Discussion

The results reported here extend the knowledge of the effects of deuterium on circadian rhythms to a new species, *Mus musculus*. Insofar as the studies parallel the one other major study done on mammals, and the few other investigations done with other organisms, the results agree quite well: the response is linear with a zero threshold. Comparing lengthening responses obtained at 30% D_2O, Suter and Rawson (1968) report a 6.45% increase, in the period of the locomotor rhythm of *Peromyscus;* Palmer and Dowse (1969) found a 6.0% lengthening of the perching activity rhythm of the waxbill, *Estrilda;* Enright (1971), a 6.33% increase in the tidal rhythm of the isopod, *Excirolana;* and (Bünning and Baltes, 1963) a 6.6% increase for the bean sleep movement rhythm. In *Mus,* the period is lengthened 7.4% by 30% D_2O ingestion.

The work presented here using blinded mice and animals maintained in DD additionally demonstrates that deuterium is not acting on the timing mechanism indirectly by altering the perception of light. It seems likely, then, that the target of deuterium lies in or very near to the clock.

Some investigators have envisioned the biological clock as one or more feedback loops, the rates of which are governed mainly by the diffusion rates of their constituents through the cell milieu. Such a model is the chronon concept (Ehret and Trucco, 1967). The period lengthening effect of heavy water certainly provides supporting evidence for such a model, as diffusion in heavy water is significantly reduced (Thomson, 1963).

Mice maintained in LD cycles and subjected to D_2O either delayed by constant intervals the phase of their locomotor rhythm, or assumed new periods, *i.e.,* appeared to ignore the ambient LD cycle and free-run. This work includes some of the first data on the effect of D_2O consumption on the entraining effectiveness of LD cycles. A preliminary report of our early findings was published in 1969 (Palmer and Dowse) and Richter (1970) has since reported in abstract, work on the hamster that supports our findings. Now, the waxbill, *Estrilda,* has also been found to respond identically (Palmer and Goodenough, unpublished experiments).

These data are in line with Aschoff's (1965) comparison of the phase relationships between organismic rhythms and LD cycles, and phase determinations of coupled physical oscillators. Using birds (Aschoff and Wever, 1962), mice (Aschoff, 1965) and lizards (Hoffman, 1968), it was shown that just as with coupled physical oscillators, the longer the period of an organism's rhythm in CC, the smaller the lead or the more the lag phase relationship when the rhythm was subjected to entraining LD cycles. In our work, we lengthened the periods of persistent rhythms with increasing concentrations of D_2O, and found that these changes manifested themselves in LD as increasing phase lags with respect to the zeitgeber cycle. As the difference between the periods of the LD cycle and rhythms was increased, a limit was eventually reached beyond which coupling was impossible; the driven rhythm then broke away and displayed its own natural frequency. In supporting Aschoff's (1965) physical-oscillator comparison, our data suggest that D_2O is acting directly at the level of the horologue complex.

Our work also shows that the increased burden placed on the zeitgeber-rhythm coupling, by increasing the natural period of the entrained rhythm with D_2O, can be counteracted by increasing the intensity of illumination during the light interval. Thus, it has been demonstrated that zeitgeber amplitude is an important factor in determining the limits within which an LD cycle can entrain a rhythm with a natural period longer than its own.

A great deal of information has been accumulated on the effects of deuterium on biological systems (for a review, see Thomson, 1963). Some of the major changes produced are: a reduction in reaction rates (*i.e.,* kinetic isotope effects), a decreased solubility of gases such as O_2 and CO_2, lowered conductivity, increased viscosity (D_2O is 18% more viscous than water at mouse body temperature), an increased acidity, a reduction in ion mobility, etc. As a result of this broad range of effects it is difficult to link a known general effect with observed rhythm perturbations.

A few possibilities suggest themselves. First, the deuterium produces an effect quite quickly: a change in period is observed within 24 hours in the waxbill and the mouse. In the isopod, Enright (1971) observed a change within a few hours after heavy water administration. Secondly, prolonged treatment does not produce larger magnitude responses, *i.e.,* the period lengthening effect is not increased

with time. By combining these two observations, it can be seen that however the chronomutagenic changes are produced, they apparently do not require substantial incorporation of deuterium into organismic compounds. It seems quite likely that much of the effect may be brought about simply by deuteration of the interstitium and the aqueous protoplasm, which, among other things, would significantly alter diffusion rates. The sameness of the responses of single- and multicellular plants and animals to deuterium shows that alterations in specific organ systems, such as nervous or endocrine systems, or so-called "blood-clock barriers" (Richter, 1970), are secondary problems.

Enright (1971) has demonstrated that D_2O decreases the output of several high frequency pacemaker systems, and since each is dependent on ion exchange, he suggests that D_2O chronomutagenicity may be a result of altered ionic flux rates. This suggestion seems reasonable.

In the study of biological rhythms, the only tangible entity with which we have to work so far is the rhythm itself; the existence of an underlying control mechanism is only deduced. To date, all attempts to locate and identify this clock have failed. In addition to the clock and the overt rhythm, a third, separate entity, a *coupler*, is also involved. The evidence for the coupler derives from a variety of

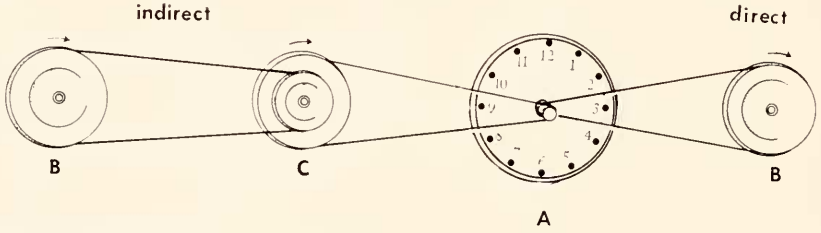


FIGURE 3. A mechanical analogy representing two means of coupling between a clock and a driven process. Details are given in the text.

experiments: a single example will suffice here. A rhythm can be inhibited for a few hours, and then, when the block is removed, the rhythm will commence again in exact phase with controls (Brown *et al.*, 1970). This clearly demonstrates that the clock is not an integral part of a rhythmic process *per se*, but is coupled to it in some way, and it is via this coupling mechanism that the necessary timing information is relayed.

Coupling may be direct or indirect; these two alternatives are represented simplistically in Figure 3, where A is the clock, B the overt driven rhythms, and C a coupler entity. The right hand paradigm illustrates direct coupling via the pulley belt, the left hand one by an interposed coupling pulley. The latter model is attractive for a number of reasons. It helps explain how an organism displaying several processes, each having a slightly different period [such as found in the oat, *Avena* (Ball and Newcomb, 1961)] could be timed by the same clock; referring to Figure 3, individual rhythms would each have its own compound coupling pulley of an appropriate size, so that each would display a different period in spite of being indirectly driven by the same clock.

Considering the possible effect of deuterium on the mechanism underlying overt

rhythms in light of the above, two interpretations are equally possible, either the clock is directly slowed, or the coupling is altered. Either would account for the D_2O-induced—or other—chronomutagenic effects. If it is the coupling that is involved and it is of the direct type, the D_2O may simply cause something analogous to a belt slippage between the driving clock and the driven pulley in Figure 3. The driving pulley (*i.e.*, the clock) would continue running at an unchanged rate, but the period of rotation of the driven pulley (*i.e.*, the timed rhythm) would be lengthened. If, however, the transfer of timing information is *via* a separate coupling entity, D_2O may cause a change in this mechanism (*e.g.*, by causing a relative change in the diameters of the intermediate compound pulley in the mechanical analogy). The net result would also be an overtly different period in the rhythm based on unchanged fundamental timing information.

Summary

The effect of deuterium oxide on the locomotor activity rhythm of the house mouse, *Mus musculus*, was measured in various environmental conditions.

1. In the initial experiments, in which mice were kept in constant low (0.2 foot candle) illumination, D_2O concentrations of 5% through 30% (in 5% increments) were administered via the drinking water. Increased concentrations lengthened the period, proportionally, with 30% D_2O causing a mean period lengthening of 7.4%.

2. Increasing the intensity of illumination in constant conditions can lengthen the period of the mouse locomotor activity rhythm. It was felt that deuterium might be changing the way in which the mice interpreted the light intensity, thus changing the period. Both blind mice, and mice kept in constant darkness were tested at various dosages. The results did not differ significantly from those obtained for mice in constant low illumination, thus showing D_2O does not act to alter the period by changing the way in which the mice interpret light intensity.

3. Concentrations of D_2O from 5% to 30% in 5% increments, were administered to mice in LD 12:12 (8). Doses up through 15% mostly caused phase shifts, at 20% some mice rephased and some were not entrained, while at the higher doses, the rhythms were not entrained to the LD cycle.

4. To test if increasing intensity of illumination in an LD cycle could diminish the perturbing influence of deuterium on the period and phase of rhythms, 20% D_2O was administered to mice in LD 12:12 (0.2), and LD 12:12 (80). The rhythms of all the mice in the first category were not entrained by the LD cycle, while all the mice in the second category were entrained. These data clearly indicate that the brighter the illumination during the light interval of an LD cycle, the stronger are its entraining capabilities of D_2O lengthened rhythms.

5. The results are discussed in terms of the bearing they have on clock models, and models for entrainment of rhythms to zeitgeber cycles.

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